Immunoblot Interpretation Criteria for Serodiagnosis of Early Lyme Disease

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We monitored the antibody responses of 55 treated patients with early Lyme disease and physician-documented erythema migrans. Six sequential serum samples were obtained from patients before, during, and until one year after antibiotic therapy and analyzed by in-house enzyme-linked immunosorbent (ELISA) and immunoblot assays. An immunoblot procedure utilizing a gradient gel and an image analysis system was developed. A relational database management system was used to analyze the results and provide criteria for early disease immunoblot interpretation. Recommended criteria for the immunoglobulin M (IgM) immunoblot are the recognition of two of three proteins (24, 39, and 41 kDa). The recommended criteria for a positive IgG immunoblot are the recognition of two of five proteins (20, 24 [>19 intensity units], 35, 39, and 88 kDa). Alternatively, if band intensity cannot be measured, the 22-kDa protein can be substituted for the 24-kDa protein with only a small decrease in sensitivity. Monoclonal antibodies were used to identify all these proteins except the 35-kDa protein. With the proposed immunoblot interpretations, the sequential serum samples were examined. At visit 1, the day of diagnosis and initiation of treatment, 54.5% of the serum samples were either IgM or IgG positive. The peak antibody response, with 80% of the serum samples positive, occurred at visit 2, 8 to 12 days into treatment. The sensitivities of the IgM and IgG immunoblot for detecting patients that were seropositive into the study period were 58.5 and 54.6%, respectively, at visit 1 and 100% at visit 2. Twenty percent of the patients remained seronegative throughout the study. The specificities of the IgM and IgG immunoblots were 92 to 94% and 93 to 96%, respectively. The IgM immunoblot and ELISA were similar in sensitivities, whereas the IgG immunoblot had greater sensitivity than the IgG ELISA ($P = 0.006$).

Lyme disease, a multisystem disorder caused by infection with the spirochete *Borrelia burgdorferi*, is the most common vector-borne disease in the United States today. The diagnosis of early Lyme disease is usually based on the presence of an expanding erythematous lesion, erythema migrans (EM). However, this clinical marker may be absent in approximately 20 to 40% of patients. Although the diagnosis is primarily based on clinical findings, it may be assisted by the results of serological tests. The enzyme-linked immunosorbent assay (ELISA) has been widely used for detecting antibodies to *B. burgdorferi*. These assays are not standardized, resulting in tests with various levels of sensitivity and specificity. Some of these tests may result in false-positive reactions, especially when sera are from persons with other illnesses such as syphilis, sarcoidosis (18), or viral illnesses (21, 26).

The Western immunoblot has also been used by investigators to study the antibody response to infection with *B. burgdorferi*, with variable results. This test has been reported to be more sensitive than ELISA for immunoglobulin M (IgM) detection (11, 14, 22) and can identify false ELISA reactions (11, 27). Karlsson et al. (15) reported that the immunoblot was more sensitive but not more specific than whole-cell ELISA in diagnosing early Lyme disease in Swedish patients with neurological involvement. In contrast, Dressler et al. (6) reported that the immunoblot can be used to increase the specificity of current serological testing for Lyme disease and have proposed interpretation criteria. However, their proposed IgM interpre-

tation criteria resulted in a low sensitivity (32%) in early disease. Aguero-Rosenfeld et al. (1) used a commercial Ig immunoblot to test patients with EM and reported that the immunoblot was more sensitive than the ELISA.

Differences in the interpretation criteria and antigen source for the immunoblot have led to confusion about the usefulness of this test for the diagnosis of Lyme disease. The difficulty of immunoblot interpretation is compounded by the problems of identification of protein bands and of defining when to consider a weak band present or absent. Some researchers discount all weak bands but fail to define intensity quantitatively (6). Densitometric studies (36) have attempted to define the significance of strong versus weak bands, yet no standard method for counting or discounting a band has been proposed.

Standardization of the methodology and interpretation of immunoblots is necessary for the effective use of this assay in the serodiagnosis of Lyme disease. We describe and compare immunoblot and ELISA results of sequential serum samples from 55 patients with early Lyme disease who presented with EM, utilizing an image analysis system and a database management system. Immunoblot results were examined statistically, and various interpretation criteria were evaluated for their sensitivity and specificity for detecting antibodies in early Lyme disease. Criteria for the interpretation of the IgM and IgG immunoblot for the serodiagnosis of early Lyme disease are proposed.

MATERIALS AND METHODS

Bacterial culture and antigen preparation. *B. burgdorferi* sensu stricto 297, a human spinal fluid isolate from Connecticut (32), was cultured at 30°C in Barbour-Stoenner-Kelly medium (2) with minor modifications (3). Low-passage 297 (fewer than 10 passages in vitro) was used for the immunoblot, and high-passage

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TABLE 1. ELISA serology results for sera from persons with early
Lyme disease, healthy blood donors, and persons
with illnesses other than Lyme disease

Sample group	Visit	n	% Positive (no. of persons positive) by:			
			IgM	IgG	IgG or IgM	IgG and IgM
Lyme disease	1	55	34.5 (19)	23.6 (13)	45.5 (25)	12.7 (7)
	2	55	63.6 (35)	41.8 (23)	76.4 (42)	29.1 (16)
	3	54	61.1 (33)	40.7 (22)	72.2 (39)	31.5 (17)
	4	54	48.1 (26)	31.5 (17)	59.3 (32)	20.4 (11)
	5	53	47.1 (24)	17.6 (9)	50.9 (27)	9.4 (5)
	6	52	12.2 (6)	8.2 (4)	19.2 (10)	1.9 (1)
Healthy donors		75	0 (0)	1.3 (1)	1.3 (1)	0.0 (0)
Other illnesses		84	12.7 (10)[a]	20.2 (17)	29.1 (23)[a]	5.1 (4)[a]

[a] $n = 79$ for this group; ELISA IgM was not tested for 5 of the 84 serum samples from persons with other illnesses.

297 (more than 50 passages in vitro) was used for the ELISA. Early-log-phase bacteria (5 to 7 days) were harvested by centrifugation at 4°C. The pellet was washed three times with ice-cold phosphate buffered saline (PBS; pH 7.2). The final suspension in 0.063 M Tris was mixed well, and aliquots (50 μl) were stored at −70°C. The protein content (micrograms per microliter) was determined with a detergent-compatible protein assay (Bio-Rad, Hercules, Calif.) according to manufacturer's instructions.

Serum samples. Six sequential serum samples per patient (55 sets, a total of 318 serum samples) were available from an early Lyme disease treatment study (23). Patients enrolled in the study had physician-documented EM and received antibiotic treatment for 20 days (500 mg of cefuroxime twice a day or 100 mg of doxycycline three times a day). Serum samples were collected at visit 1 (V1, the day treatment was initiated), V2 (day 8 to day 12 into treatment), V3 (day 1 to day 5 posttreatment [PT]), V4 (day 30 PT), V5 (day 90 PT), and V6 (1 year PT). All except five patients responded satisfactorily to antibiotic treatment as assessed at V4. Serum samples from healthy donors (normals) were collected by the Red Cross from an area of nonendemicity for Lyme disease ($n = 75$). Potentially cross-reactive samples ($n = 84$) were collected from patients with rheumatoid arthritis ($n = 16$), systemic lupus erythematosus ($n = 5$), multiple sclerosis ($n = 10$), syphilis ($n = 29$), relapsing fever ($n = 2$), infectious mononucleosis ($n = 12$), leptospirosis ($n = 6$), and group A streptococcal sequelae ($n = 4$).

MAbs. Monoclonal antibodies (MAbs) were used to identify proteins of *B. burgdorferi* 297. H9724 (flagellin), H5332 (OspA), H5TS (OspB), and P39 (39 kDa) were kindly provided by T. Schwan, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.; CB312 (DnaK), CB625 (22 kDa), and CB49 (19 kDa) were provided by J. Benach, New York Department of Health, Stony Brook; P20a (22 kDa) and O62a (62 kDa) were provided by T. Masuzawa, University of Shizuoka, Shizuoka, Japan; D4 (82 to 93 kDa) was provided by D. Volkman, State University of New York, Stony Brook; L22 1F8 (OspC) was provided by B. Wilske, Pettenkofer-Institut, University of Munich, Munich, Germany; 86 DN-1 (25 kDa) was provided by P. Duffey, Department of Health Services, Berkeley, Calif.; 184.1 (OspA, 22 kDa) was provided by B. Luft, State University of New York, Stony Brook; and 240.7 (7.5 kDa) was provided by G. Habicht, State University of New York, Stony Brook.

ELISA. ELISA was performed as described previously (10) on Lyme disease samples (55 on V1, 55 on V2, 54 on V3, 54 on V4, 53 on V5, and 52 on V6), healthy blood donors, and persons with other illnesses. Briefly, a whole cell sonicate filtrate of *B. burgdorferi* 297 was the antigen source. The 96-well microtiter plates were coated with 0.5 μg of protein per well of the sonicate filtrate (0.22 μm) and dried overnight uncovered at 37°C. The plates were blocked with 1% horse serum, washed four times with 0.05% Tween 20–PBS, incubated with a 1:200 dilution of patient serum for 1 h, and washed five times with 0.05% Tween 20–PBS. After this, the wells were incubated with horseradish peroxidase-conjugated anti-human IgM or IgG, washed five times, and incubated with peroxidase substrate, and the optical density was determined at 405 nm. An optical density of 3 standard deviations or greater above the mean optical density of 200 serum specimens from Red Cross healthy blood donors was considered positive. Between two and three standard deviations above the same mean was considered borderline. Less than two standard deviations above the mean was considered negative.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (13). Briefly, proteins were separated with a linear gradient polyacrylamide gel (7.5 to 15%) (12) with a thickness of 0.75 mm. The gels were poured in a multiple gel caster (Hoefer

Scientific Instruments, San Francisco, Calif.) and stored at 5°C for up to 1 month. On the day of electrophoresis, a 3.75% stacking gel was poured with a comb with a 12.2 cm trough and one lane (6 mm). A cell suspension of low passage *B. burgdorferi* 297 was thawed, diluted appropriately in sample buffer consisting of 0.063 M Tris, 2% SDS, 15 mM dithiothreitol, 27% sucrose, and 0.002% bromophenol blue, and boiled for 2 min. Three hundred micrograms of protein was loaded onto the gel, and 2 μl of low-molecular-weight standards (Pharmacia LKB) was loaded into the single lane. Electrophoresis by the discontinuous buffer system of Laemmli (16) was carried out at a 35-mA constant current per gel at room temperature for approximately 1.5 h until the dye front reached the bottom of the gel. Following electrophoresis, proteins were transferred to Immobilon P (Millipore Corp., Bedford, Mass.) at 1 A for 30 min by the method of Towbin et al. (33). The standard lane and the sides of the trough were cut off the membrane, washed, and stained with India ink. The center of the membrane containing the antigen section was dried on filter paper and stored in a dry dark place for up to 4 weeks at room temperature.

Western immunoblot. Human serum samples, including those from Lyme disease patients (55 on V1, 55 on V2, 54 on V3, 54 on V4, 51 on V5, 49 on V6), from healthy blood donors, and from persons with other illnesses, were examined by immunoblot for reactivity to proteins of *B. burgdorferi* 297. Two serum samples from V5 and three samples from V6 tested by ELISA were unavailable for immunoblot testing. Prior to immunoblot, the antigen section was marked at the top and bottom, with the India ink-stained sections as guides, for later trimming. A 10.6-cm height, which contained proteins ranging from less than 10 kDa to greater than 150 kDa, was kept. The antigen section was rewet with 0.5% Tween 20–PBS for 25 min. The blot was then blocked for 1 h with 0.5% instant nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) and washed for 45 min with 0.1% Tween 20–TBS. After being blocked, the blot was trimmed at the markings on the top and bottom. The remaining section was cut into 3- to 4-mm strips (approximately 10 to 11 μg of protein per strip), which were numbered and incubated for 1 h with human serum samples at a dilution of 1:400 (5 μl of serum plus 2 ml of 0.5% instant nonfat dry milk in TBS) in trays (Accutran disposable incubation trays; Schleicher and Schuell, Keene, N.H.). The strips were then washed twice for 5 min each with 0.1% Tween 20–TBS, after which they were incubated with 2 ml of alkaline phosphatase-conjugated goat anti-human IgG (1:6,000) or IgM (1:5,000) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in 0.5% instant nonfat dry milk in TBS for 1 h. The strips were washed twice for 5 min with 0.1% Tween 20–TBS, twice for 5 min with TBS, and twice for 5 min with barbital buffer (150 mM sodium barbital [pH 9.6]). Color was developed for 20 min at 22 to 23°C with a developing solution of 300 μg of nitroblue tetrazolium per ml, 150 μg of 5-bromo-4-chloro-3-indolylphosphate per ml, and 813 μg of $MgCl_2 \cdot 6H_2O$ per ml in barbital buffer. Control of immunoblot intensity development time was kept consistent by the use of moderate IgM- and IgG-positive human control sera with band reactivities of various intensities. Strongly positive control sera were unsatisfactory for this purpose because color development occurred too quickly and led to difficulty in assessing color development with sera containing lower levels of antibodies. To facilitate easier and more accurate band identification, control strips were placed at the first, middle, and last strips of the immunoblot.

Immunoblot analysis. A video optical scanning system consisting of a video camera (500 lines resolution; Panasonic WVBL600), videodigitizing circuit, microcomputer (MacIntosh IIci), Image public domain software (20, 29), and National Center for Supercomputing Applications (NCSA) GelReader software were utilized. NCSA GelReader was used to obtain the molecular weight and intensity of each protein on each immunoblot strip. The intensity was set on a gray scale of 1 to 256 units. The peak of each band was selected for the intensity reading. Three positive IgG control strips on each blot were used to identify nine different proteins of *B. burgdorferi* (88, 72, 58, 41, 39, 34, 31, 24, and 20 kDa), which were used as size markers. These nine proteins had been previously identified with MAbs. The lowest intensity reading (background) level on each blot was set as 1 intensity unit, and all other readings were adjusted accordingly.

Band and intensity information and ELISA serology results for all patient samples were stored in an Ingres relational database management system. This database management system was used because its querying capabilities allowed immunoblot band patterns common to a set of patients to be extracted from the database and summarized.

Statistical analysis. To determine which proteins were significant markers for early Lyme disease, the frequencies of reactivity for each protein with Lyme disease sera or normal sera were compared in 2-by-2 contingency tables by χ^2 analysis.

RESULTS

We examined sequential serum samples from 55 patients with early Lyme disease and physician-documented EM by ELISA and immunoblot. This patient set was selected from a larger group of 123 patients from a 1-year clinical antimicrobial trial (23). The sample set was not randomly chosen, since patients with serum samples available from each of the six time points (V1 to V6) were more likely to be included in the study.

TABLE 2. ELISA and immunoblot results for persons with illnesses other than Lyme disease

Illness	n	No. positive by[a]:			
		IgM ELISA	IgG ELISA	IgM immunoblot[b]	IgG immunoblot[c]
Syphilis	29	3[d]	15	0	0
Rheumatoid arthritis	16	1 (25)	0	1 (25)	1 (25)
Infectious mononucleosis	12	4 (23, 24, 65, 67)	0	2 (24, 67)	0
Multiple sclerosis	10	0	0	0	2 (00, 02)
Leptospirosis	6	1 (62)	0	0	1 (61)
Systemic lupus erythematosus	5	0	0	1 (36)	1 (35)
Group A strep sequelae	4	0	0	0	0
Relapsing fever	2	1 (68)	2 (68, 69)	1 (68)	1 (69)

[a] Numbers in parentheses are individual patient identification numbers.
[b] According to the positive IgM immunoblot interpretation criterion two of three proteins (41, 39, and 24 kDa).
[c] According to the positive IgG immunoblot interpretation criterion two of five proteins (88, 39, 35, 24 [>19 intensity units], and 20 kDa).
[d] All three syphilis patients testing ELISA IgM positive were also IgG ELISA positive.

ELISA. Prior to treatment (day treatment was initiated at V1), 25 of the 55 patients (45.5%) had a positive IgM or IgG antibody response by ELISA (Table 1). Twelve (21.8%) of the patients had a positive response only for IgM, and six (10.9%) patients had a positive response only for IgG. Seven patients (12.7%) were positive by both IgM and IgG ELISA. Seven patients (12.7%) had either a borderline IgM or a borderline IgG response, and 23 (41.8%) had a negative response at V1). After 8 to 12 days of treatment (V2), 32.7% (18 of 55) of patients seroconverted to either a positive IgM or a positive IgG ELISA result. The percent of patients with a positive IgM or IgG ELISA response increased from 45.5% (25 of 55) at V1 to 76.4% (42 of 55) at V2, and this was the maximum number of patients with a positive IgM or IgG ELISA response during the 1-year study (Table 1). Also, between V1 and V2 the percent of patients with both a positive IgM response and a positive IgG response increased from 12.7% (7 of 55) to 29.1% (16 of 55). After V2, the percent of patients with a positive IgM or IgG response declined with each subsequent visit, reaching a low of 19.2% (10 of 52) at V6, 1 year PT. At 1 year PT, 12.2% (6 of 52) of the patients were IgM positive and 8.2% (4 of 52) were IgG positive. Twenty percent (11 of 55) of the patients did not respond with either a positive IgM or a positive IgG ELISA at any of the six visits.

Only one of 75 healthy blood donors had a positive IgG ELISA result, and none had a positive IgM ELISA result (Table 1). However, persons with illnesses other than Lyme disease were more likely than healthy blood donors to have positive ELISA results. Of persons with other illnesses, 12.7% (10 of 79) had positive ELISA IgM results, including three with syphilis, one with rheumatoid arthritis, four with infectious mononucleosis, one with leptospirosis, and one with relapsing fever (Table 2). Twenty percent (17 of 84) of persons with other illnesses had a positive IgG ELISA. Fifteen of these patients had syphilis, and two patients had relapsing fever.

Immunoblot. We next investigated the use of the immunoblot in combination with the image analysis system to monitor the antibody response in early Lyme disease. MAbs were used to identify the location of various proteins of *B. burgdorferi* 297 separated by a 7.5 to 15% linear gradient polyacrylamide gel (Table 3). With this gradient gel, the 39-kDa protein was clearly resolved from the 41-kDa flagellin protein (Fig. 1). The following proteins were identified with MAbs and used as reference markers for the immunoblot: 88, 72, 58, 41, 39, 34, 31, 24, and 20 kDa. By the image analysis system and with moderately positive IgG control serum, the mean intensity reading for each of the nine reference proteins on each immu-

noblot was calculated. The 88-kDa protein had the lowest average intensity reading, 15 U, and the 39-kDa protein had the highest average intensity reading, 92 U. A standard deviation was calculated for the intensity readings of each reference protein and used to assess the reproducibility of this assay. The assay was very reproducible, with only 1 of the 43 immunoblots having two reference proteins with an intensity reading greater than 3 standard deviations from their mean intensity readings. Of the 43 immunoblots, 6 had one of the nine reference proteins outside of 3 standard deviations.

The number of *B. burgdorferi* 297 proteins reactive with sera of early Lyme disease patients was determined for each of the six visits (Table 4). Although the standard deviation is large, the pattern observed was an increase in the number of protein bands reactive with IgM and IgG from V1 (5.8 and 6.7, respectively) to V2 (9.3 and 8.7, respectively) followed by a gradual decrease in the number of reactive protein bands to a low of 4.3 on IgM and 6.7 on IgG at V6. Lower numbers of reactive protein bands were present in immunoblots of healthy blood donors and patients with other illnesses (Table 4). The numbers of IgM- and IgG-reactive bands were 3.0 and 4.1 for healthy blood donors and 2.1 and 3.7 for patients with other illnesses, respectively.

The frequencies at which the various proteins of *B. burgdorferi* 297 were reactive with the sera of ELISA-positive early Lyme disease patients were compared with those of normal

TABLE 3. Protein location as determined by MAb reactivity

MAb	MAb specificity[a]	Size of our corresponding blot protein (kDa)
D4	82–93	88
CB312	DNaK	72
O62a	62	58
H9724	41	41
P39	39	39
H5TS	OspB	34
H5332	OspA	31
86 DN-1	25	NR[b]
L22 1F8	OspC	24
184.1	OspA, 22	31
CB625	22	22
P20a	22	20
CB49	19	20
240.7	7.5	20, 10

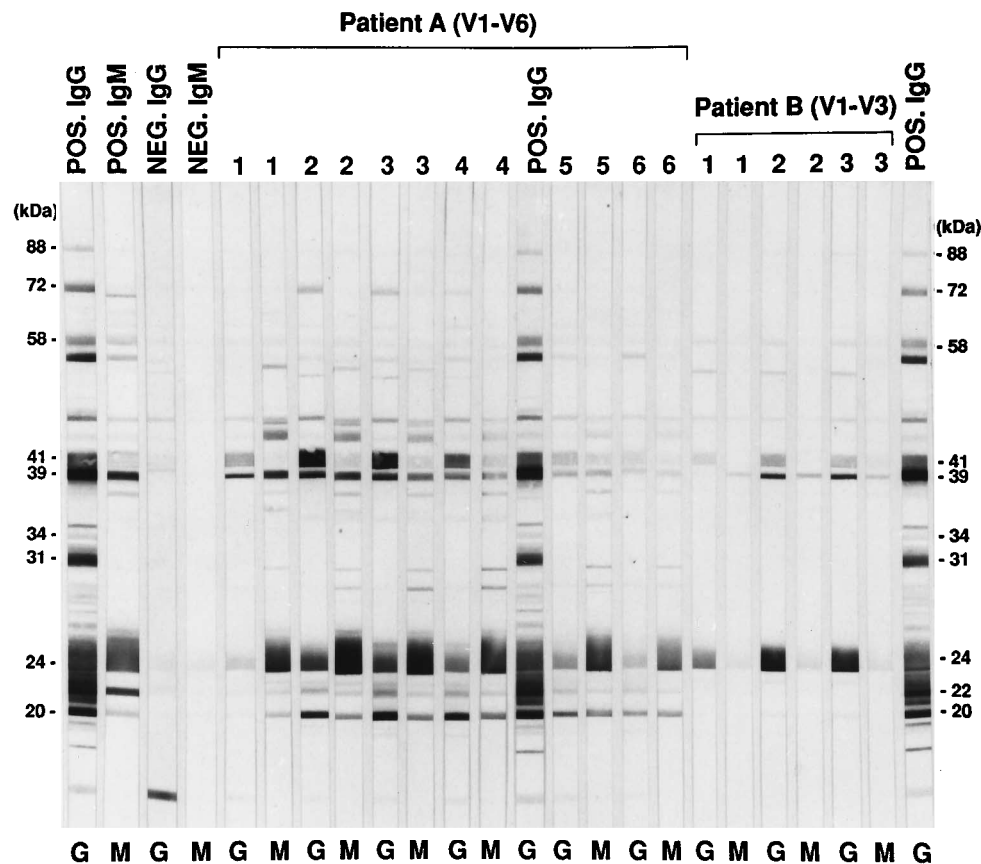[a] Proteins are identified by size (in kilodaltons) or by name.
[b] NR, nonreactive.

FIG. 1. A representative immunoblot of serum samples from two patients with early Lyme disease. All six visits are shown for patient A, and V1 to V3 are shown for patient B. The corresponding ELISA results for patient A are V1, IgM positive and IgG negative; V2, IgM positive and IgG positive; V3, IgM positive and IgG positive; V4, IgM positive and IgG negative; V5, IgM positive and IgG negative; and V6, IgM positive and IgG negative. For patient B, the corresponding ELISA results are V1, IgM negative and IgG negative; V2, IgM positive and IgG negative; and V3, IgM positive and IgG negative. Markers are identified in kilodaltons on the left and right sides. On the bottom of the figure, G indicates IgG and M indicates IgM. On the top of the figure, numbers are patient visit numbers (V1 to V6). Control sera are labeled as positive IgM, positive IgG, negative IgM, and negative IgG.

blood donors by χ^2 analysis. Arbitrarily, proteins reactive with samples from fewer than 35% of ELISA-positive early Lyme disease patients were not studied further. The frequencies of recognition of *B. burgdorferi* 297 proteins by IgM and IgG of ELISA-positive early Lyme disease patients (V1 and V2) and healthy blood donors are shown in Tables 5 and 6. Proteins of significance ($P < 0.01$) for the IgM immunoblot include the following, in decreasing order of significance: 39, 41, 23, 44, 88, 56, 24, 37, 20, 46, and 58 kDa (Table 5). The 39-kDa protein, in addition to being the most significant protein, was also the protein most frequently reactive on IgM immunoblot for patients with early Lyme disease. This protein was recognized by 84% of samples from these patients at V1 and by 94% at V2. In contrast, only 6.7% of the healthy blood donors' samples were reactive with the 39-kDa protein.

Eight proteins were found to be significant for the IgG immunoblot in early Lyme disease. In decreasing order of significance, they are 39, 20, 35, 22, 56, 72, 23, and 88 kDa. Again, the 39-kDa protein was the most significant, and early Lyme disease patients' sera reacted with this protein with a high frequency.

Some proteins were recognized by healthy blood donors frequently but at a very low intensity (weak band), while showing a more intense reaction with sera of early Lyme disease patients. We examined the possibility that the statistical significance of these proteins could be increased by establishing an

intensity reading cutoff that would discount reactivities at low background levels (Table 7). Utilizing an intensity reading cutoff of greater than 19 U proved useful for three proteins (46, 41, and 24 kDa) on IgG immunoblot. OspC (24 kDa) was frequently recognized at a low intensity by sera of many healthy blood donors (49%, 37 of 75) but only by 5.3% (4 of 75) with an intensity reading of greater than 19 U. Similar results were seen for healthy blood donors with respect to

TABLE 4. Average numbers of bands seen on immunoblot

Group	Visit	No. of bands of indicated type (mean ± SD)	
		IgM	IgG
Lyme disease	1	5.8 ± 6.3	6.7 ± 4.1
	2	9.3 ± 7.1	8.7 ± 4.1
	3	8.1 ± 6.2	8.6 ± 4.2
	4	6.5 ± 5.4	8.2 ± 4.1
	5	5.3 ± 4.8	7.4 ± 4.0
	6	4.3 ± 3.7	6.7 ± 3.9
Healthy donors		3.0 ± 2.6	4.1 ± 2.6
Other illnesses		2.1 ± 3.0	3.7 ± 2.3

TABLE 5. Frequency of recognition of proteins of *B. burgdorferi* 297 by IgM of ELISA-positive early Lyme disease sera and healthy donor sera

| Protein[a] | Frequency (% reactive) of recognition by IgM of: | | | P value[b] |
| | Early Lyme disease sera[c] | | Sera of healthy human donors ($n = 75$) | |
	V1 ($n = 19$)	V2 ($n = 35$)		
88*[d]	42	49	2.7	6.4×10^{-7}
58*	48	54	17	0.0057
56*	32	35	1.3	7.3×10^{-6}
46*	74	83	37	0.0044
44*	42	37	1.3	6.9×10^{-8}
41*	68	77	9.3	1.9×10^{-8}
39*	84	94	6.7	4.2×10^{-13}
37*	53	80	19	0.0024
35	47	63	24	0.0443
24*	84	91	44	0.0017
23*	37	40	0.0	4.7×10^{-8}
22	42	57	29	0.2861
20*	68	77	31	0.0025

[a] Identified by size in kilodaltons.
[b] Sera from healthy human donors were compared with early Lyme disease sera from V1.
[c] Data shown are for proteins having frequencies (percent reactive) of at least 35% at V1 or V2 and for sera positive by IgM ELISA.
[d] *, statistically significant for early Lyme disease ($P < 0.01$).

flagellin (41 kDa) and the 46-kDa protein, for which *P* values were decreased to less than 0.01 with an intensity reading cutoff of greater than 19 U. Intensity reading cutoffs were not useful for any proteins on IgM immunoblot.

Using proteins that were statistically significant on the basis of comparison with those of healthy blood donors for IgM and IgG immunoblot, we examined the numbers of these proteins

TABLE 6. Frequency of recognition of proteins of *B. burgdorferi* 297 by IgG of ELISA-positive early Lyme disease sera and healthy donor sera

| Protein[a] | Frequency (% reactive) of recognition by IgG of: | | | P value[b] |
| | Early Lyme disease sera[c] | | Sera of healthy human donors ($n = 75$) | |
	V1 ($n = 13$)	V2 ($n = 23$)		
88*[d]	23	35	5.3	1.6×10^{-4} (V2)
72*	31	44	6.7	0.0081
58	54	70	45	0.5700
56*	39	44	5.3	2.7×10^{-4}
46	100	70	71	0.0241
41	85	91	49	0.0183
39*	85	91	4.0	2.2×10^{-13}
35*	46	57	9.3	5.5×10^{-4}
24	77	83	49	0.0656
23*	31	35	0.0	8.8×10^{-7}
22*	46	52	9.3	5.5×10^{-6}
20*	69	83	13	1.9×10^{-10}

[a] Identified by size in kilodaltons.
[b] Sera from healthy human donors were compared with early Lyme disease sera from V1, unless specified otherwise.
[c] Data shown are for proteins having frequencies (percent reactive) of at least 35% at V1 or V2 and for sera positive by IgG ELISA.
[d] *, statistically significant for early Lyme disease ($P < 0.01$).

TABLE 7. Use of intensity reading cutoffs to increase the statistical significance of some proteins of *B. burgdorferi* 297 for Lyme disease on IgG immunoblot

| Protein[a] | % of samples of indicated type found reactive at indicated intensity reading cutoff | | | | P value[b] |
| | Early Lyme disease sera (V1)[c] | | Sera of healthy human donors | | |
	All levels	>19 U	All levels	>19 U	
46	100	77	71	11	4.6×10^{-8}
41	85	62	49	19	9.8×10^{-4}
24	77	46	49	5.3	1.9×10^{-5}

[a] Identified by size in kilodaltons.
[b] The *P* value was calculated by comparison of early Lyme disease and healthy serum reactivities, with intensity reading cutoffs set at >19 U.
[c] IgG ELISA positive; $n = 13$.

with which samples from Lyme disease patients reacted regardless of the pattern of reactivity (Table 8). We found interpretation criteria of this type to be useful when the early Lyme disease group was compared with healthy blood donors (data not shown) but not useful in comparisons with persons with illnesses other than Lyme disease. For example, at V1, 63.2% of ELISA IgM-positive early Lyme disease serum samples reacted with any 5 of the 11 significant proteins for IgM immunoblot, but only 30.9% of all early Lyme disease patients reacted with any 5 significant proteins. This criterion was not sensitive, although it was specific for early Lyme disease as only 6.0% of persons with other illnesses reacted. Similar results were found for IgG immunoblot with any 4 of 11 significant proteins, such that 69.2% of ELISA IgG-positive early Lyme disease serum samples and 36.4% of all early Lyme disease serum samples at V1 reacted and only 8.3% of samples from persons with other illnesses reacted.

Since the above criteria could provide specificity but lacked a high sensitivity, we examined the reactivity of specific combinations of significant bands of *B. burgdorferi* to establish the most sensitive and specific interpretation criteria for serodiagnosis of early Lyme disease (Table 9). The results of this investigation showed that for the IgM response, the most sensitive and specific criterion was recognition of two of three bands (41, 39, and 24 kDa). When this criterion was used, 43.6% (24 of 55) of early Lyme disease patients were positive at V1 (the day treatment was initiated) and 74.5% (41 of 55) were positive at V2 (8 to 12 days of treatment). In fact, eight patients having negative IgM ELISA results at V1 were positive by IgM immunoblot according to these criteria. Only one patient converted on IgM immunoblot from negative to positive after V2, compared with 17 patients converting between V1 and V2. This IgM interpretation pattern was specific (92 to 94%) for early Lyme disease, since only 8% (6 of 75) of healthy blood donors and 6.0% (5 of 84) of persons with illnesses other than Lyme disease were positive. Persons with other illnesses recognizing two of three (41, 39, and 24 kDa) proteins on IgM immunoblot included two with infectious mononucleosis and positive ELISA IgM serology, one with rheumatoid arthritis and positive ELISA IgM serology, one with relapsing fever and positive ELISA IgM and IgG serology, and one with systemic lupus erythematosus and negative ELISA serology (Table 2). Although not statistically significant, the IgM immunoblot interpretation criteria appeared to be more sensitive in detecting antibodies in early Lyme disease than IgM ELISA since at V1 43.6% (24 of 55) of patients were positive by IgM immunoblot versus 34.5% (19 of 55) positive by ELISA IgM. This same

TABLE 8. Numbers of significant *B. burgdorferi* proteins recognized on immunoblot by sera of persons
with early Lyme disease or other illnesses

| No. of significant proteins recognized[a] | Frequency (% reactive) of recognition by Ig of indicated type of serum samples from donors with disease of indicated type | | | | | |
| | IgM | | | IgG | | |
	Early Lyme disease (V1; $n = 55$)	ELISA IgM positive early Lyme disease (V1; $n = 19$)	Other illnesses ($n = 84$)	Early Lyme disease (V1; $n = 55$)	ELISA IgG positive early Lyme disease (V1; $n = 13$)	Other illnesses ($n = 84$)
2	72.7	94.7	21.4	61.8	84.6	50.0
3	50.9	84.2	13.1	45.5	84.6	22.6
4	38.2	80.0	11.9	36.4	69.2	8.3
5	30.9	63.2	6.0	25.5	53.8	4.8
6	23.6	63.2	3.6	18.2	53.8	2.4
7	21.8	63.2	2.4	16.4	46.2	1.2
8	14.5	42.1	1.2	12.7	38.5	0.0

[a] Significant IgM proteins include 88, 58, 56, 46, 44, 41, 39, 37, 24, 23, and 20 kDa; significant IgG proteins include 88, 72, 56, 46 (>19 U), 41 (>19 U), 39, 35, 24 (>19 U), 23, 22, and 20 kDa.

trend was seen at V2, when 74.5% (41 of 55) of early Lyme disease patients were IgM immunoblot positive versus 63.6% (35 of 55) positive by ELISA IgM.

The most sensitive and specific interpretation criterion for IgG immunoblot in early Lyme disease was found to be the recognition of two of the following five proteins: 88, 39, 35, 24 (>19 intensity units), and 20 kDa. According to this criterion, 43.6% (24 of 55) of Lyme disease patients were positive by immunoblot at V1 versus 23.6% (13 of 55) positive by ELISA IgG ($P = 0.026$). At V2, 80.0% (44 of 55) of early Lyme disease patients were IgG immunoblot positive compared with 41.8% (23 of 55) positive by IgG ELISA ($P < 0.001$). Few healthy blood donors or persons with other illnesses tested IgG immunoblot positive with this IgG interpretation criterion (4.0 and 7.1%, respectively). Persons with other illnesses testing IgG immunoblot positive included two with multiple sclerosis and negative ELISA serology, one with systemic lupus erythematosus and negative ELISA serology, one with leptospirosis and negative ELISA serology, one with relapsing fever and positive ELISA IgG serology, and one with rheumatoid arthritis and

positive IgM ELISA serology (Table 2). Overall, one patient with rheumatoid arthritis had both IgM and IgG immunoblot and ELISA tests positive.

Since the IgG immunoblot criterion requires an image analysis system to analyze the intensity of the 24-kDa protein, it may not be useful to laboratories without this ability. We found that recognition of two of the five proteins 88, 39, 35, 22, and 20 kDa was only slightly less sensitive than the previously mentioned IgG immunoblot interpretation criterion. This pattern replaces recognition of the 24-kDa protein at an intensity greater than 19 U with recognition of the 22-kDa protein at any intensity. According to this IgG immunoblot interpretation, 40% (22 of 55) and 76.4% (42 of 55) of V1 and V2 serum samples were positive, respectively. This compares favorably with the previous pattern incorporating intensity measurements, according to which 43.6% (24 of 55) and 80% (44 of 55) of serum samples at V1 and V2 were positive. According to this modified IgG immunoblot interpretation, one additional patient with other illnesses became positive, resulting in an 8.3% (7 of 84) reactivity. No difference in the numbers of healthy

TABLE 9. Use of patterns for interpretation of IgM and IgG immunoblots

| Group | Visit | ELISA result[a] | % Immunoblot positive (no. positive/no. tested) | | |
			IgM[b]	IgG[c]	IgM or IgG[b,c]
Early Lyme disease	1	Pos., bord., or neg.	43.6 (24/55)	43.6 (24/55)	54.5 (30/55)
	1	Pos.	84.2 (16/19)	76.9 (10/13)	NA[d]
	1	Bord.	0.0 (0/1)	66.7 (2/3)	NA
	1	Neg.	22.9 (8/35)	30.8 (12/39)	NA
	2	Pos., bord., or neg.	74.5 (41/55)	80.0 (44/55)	80.0 (44/55)
	2	Pos.	97.1 (34/35)	91.3 (21/23)	NA
	2	Bord.	66.7 (4/6)	90.0 (9/10)	NA
	2	Neg.	21.4 (3/14)	63.6 (14/22)	NA
	3	Pos., bord., or neg.	72.2 (39/54)	79.6 (43/54)	80.0 (43/54)
	4	Pos., bord., or neg.	59.3 (32/54)	77.8 (42/54)	80.0 (43/54)
	5	Pos., bord., or neg.	41.2 (21/51)	68.6 (35/51)	72.5 (37/51)
	6	Pos., bord., or neg.	26.5 (13/49)	49.0 (24/49)	59.2 (29/49)
	1–2	Pos., bord., or neg.	74.5 (41/55)	80.0 (44/55)	NA
	1–4	Pos., bord., or neg.	76.4 (42/55)	81.8 (45/55)	NA
Healthy donors		Pos., bord., or neg.	8.0 (6/75)	4.0 (3/75)	12.0 (9/75)
Other illnesses		Pos., bord., or neg.	6.0 (5/84)	7.1 (6/84)	11.9 (10/84)

[a] Refers to corresponding ELISA IgM or IgG results of samples; pos., positive; bord., borderline; neg., negative.
[b] Positive interpretation criterion: two of three proteins (41, 39, and 24 kDa).
[c] Positive interpretation criterion: two of five proteins (88, 39, 35, 24 [>19 intensity units], and 20 kDa).
[d] NA, not analyzed.

blood donors with positive IgG immunoblots results was observed with these two interpretations.

DISCUSSION

Increased use of the immunoblot has resulted from some of the uncertainties associated with the results obtained with the variety of ELISA and indirect immunofluorescent assays presently available. The immunoblot has the potential of being more sensitive and specific than the ELISA and indirect immunofluorescence assay and has been used to confirm results obtained with these assays. However, the lack of standardization of antigen preparations, techniques, and interpretation has limited its usefulness. In an attempt to improve the sensitivity and specificity of the immunoblot, we used a linear gradient gel for better resolution of proteins and an image analysis system and database management system to develop interpretation criteria. The availability of six sequentially collected serum specimens before, during, and after antibiotic treatment from 55 patients with early Lyme disease (physician-documented EM) provided the opportunity to monitor the antibody profiles of these patients in detail.

We first determined which proteins of *B. burgdorferi* 297 that reacted with the antibodies of patients with early Lyme disease were significant. Next, we examined several interpretation criteria for the immunoblot, utilizing our relational database management system. We found that the number of significant proteins a person's serum reacted with was not generally useful since the number of reactive bands required for good specificity resulted in a low level of sensitivity. The immunoblot interpretation recommended by Dressler et al. (6) and used by Aguero-Rosenfeld et al. (1) advanced the following criteria for positive immunoblots: for the IgM immunoblot, at least 2 of 8 common bands in early disease (18, 21, 28, 37, 41, 45, 58, and 93 kDa) and for the IgG immunoblot, at least 5 of 10 bands (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa) after the first weeks of infection. It was not possible for us to accurately match a number of our protein bands with those described by Dressler et al. (6). For example, we found that our 88- and 24-kDa proteins correspond to their 93- and 21-kDa proteins. However, we were uncertain as to which of our proteins correspond to their 18-, 30-, and 45-kDa proteins. Accordingly, we were not able to satisfactorily apply their interpretation criteria to our immunoblots. This difficulty in matching protein bands is probably due to the use of different strains of *B. burgdorferi* as the antigen source and the use of different acrylamide gel concentrations for protein separation. It also emphasizes the importance of using MAbs for identification of protein bands.

Our study of the use of the immunoblot for the serodiagnosis of early Lyme disease demonstrated that relatively simple criteria can be used for the interpretation of IgM and IgG immunoblots. Only two of three proteins (24 [OspC], 39, and 41 kDa) need be recognized for a positive IgM immunoblot. Recognition of just two of five proteins (20, 24 [>19 intensity units], 35, 39, and 88 kDa) will satisfy the requirements for a positive IgG immunoblot. If a laboratory is not equipped to measure intensity, then the 22-kDa protein can be substituted for the 24-kDa protein. This substitution only slightly decreases the sensitivity of the IgG immunoblot. In addition to the requirement under the proposed interpretation for the identification of only 6 or 7 proteins, MAbs are available for all the proteins except the 35 kDa. As discussed previously, the use of MAbs is of critical importance for the standardization of the immunoblot assay. On the basis of the analysis of a limited number of serum specimens, we found that the criterion we developed for the positive IgG immunoblot in early Lyme

disease could also be applied to late Lyme disease. Although antibodies to the 31-kDa (OspA) and 34-kDa (OspB) proteins occur relatively infrequently, these proteins can be included among the significant proteins reactive in late disease because of their high levels of specificity, especially when they occur in tandem. In addition, MAbs for identification of these proteins are available.

The flagellin protein (41 kDa) has been shown to be one of the first proteins that antibodies are directed against after infection with *B. burgdorferi* (4, 5, 11, 17, 22, 36). We found the flagellin protein to be significant for the IgM immunoblot. It was not significant for the IgG immunoblot unless an intensity reading cutoff was used. This was due to the presence of low levels of IgG directed against the flagellin in sera from healthy blood donors and persons with illnesses other than Lyme disease. However, the inclusion of the 41-kDa protein with an intensity cutoff in the criterion for a positive IgG immunoblot did not improve the specificity or sensitivity of the proposed interpretation. Similar results were observed with the 46-kDa protein, which required an intensity level cutoff in order to be significant for the IgG immunoblot.

Another dominant antibody response in early Lyme disease is directed against OspC. The European investigators Wilske et al. (35) and Fuchs et al. (8) were the first to describe and characterize this immunodominant antigen, originally referred to as pC. North American investigators have also described an early and dominant antibody response to this protein in patients with Lyme disease (1, 6, 9, 24). However, the expression of this protein is variable, and it may be poorly expressed in strains that have been passaged many times in vitro (19, 25, 28, 34). We have used low-passage strain 297 for our immunoblot studies, because OspC is the major protein expressed whereas our high-passage 297 expresses this protein at a low level. Using this low passage strain, we observed a strong, specific, and frequent IgM response to the 24-kDa protein (OspC). The IgG response to OspC was also strong and frequent in patients with early Lyme disease, but a response also occurred at a low intensity in healthy blood donors and persons with other illnesses. In order to include OspC as a significant reactive protein for the IgG response, it was necessary to use an intensity reading cutoff of greater than 19 U.

Simpson et al. (31) reported that the 39-kDa protein is specific for *B. burgdorferi* and is strongly serologically reactive. Ma et al. (17) found the 39-kDa protein to be the most significant marker for Lyme borreliosis, with approximately 50% of serum samples from patients with early disease reacting with this protein. Aguero-Rosenfeld et al. (1) reported that antibodies against the 39-kDa protein were observed in 35% of IgM and 26% of IgG immunoblots during the acute phase of early disease. In contrast, Dressler et al. (6) did not observe antibodies reactive with the 39-kDa protein in patients with early Lyme disease. We found the 39-kDa protein to be the most common specific marker for early Lyme disease on both IgM and IgG immunoblots. These variable results could be attributed to the lack of resolution of the 41- and 39-kDa proteins in gel concentrations of less than 12.5%. Variations in the expression of this protein in the strains used may also be important.

We found that the immunoblot was both very sensitive and specific for detecting antibodies in patients with early Lyme disease. Results from our proposed criteria for immunoblot interpretation were compared with results from our in-house ELISA. We found the immunoblot to be more specific than the ELISA. In our control group of 84 serum specimens from patients with illnesses that might resemble Lyme disease, the specificities of the IgM ELISA and the IgG ELISA were 87

and 80%, respectively, whereas the specificities of the IgM and IgG immunoblots were 94 and 93%, respectively ($P = 0.006$). The IgM ELISA and immunoblot were similar in sensitivity for detecting antibodies in early Lyme disease. Although the IgM immunoblot was slightly more sensitive than the IgM ELISA, the difference was not statistically significant. There was a significant difference between the sensitivities of the IgG ELISA and immunoblot. At V1 and V2 (acute- and convalescent-phase specimens), 34.5 and 63.6% of the patients were ELISA IgG positive whereas 43.6 and 80.0% were positive by IgG immunoblot. The finding that the IgG immunoblot was already positive for 80.0% of the patients after only 8 to 12 days of treatment was unexpected. Generally, the IgG response at this early stage of the disease was thought to be of little diagnostic value. For example, Dressler et al. (6) reported that their 25 patients with EM had only a minimal specific Ig response 2 to 4 weeks after antibiotic therapy and did not analyze these serum samples with the IgG immunoblot. Our results suggest that the IgG response can be of considerable value in the serodiagnosis of early Lyme disease. In addition, some patients who are reinfected may have an IgG response only in early disease. It is possible that the greater sensitivity of the immunoblot versus the ELISA may be due in part to the use of high-passage *B. burgdorferi* 297 in the ELISA and low-passage 297 in the immunoblot.

The highest level of positive serology was seen at V2 (day 8 to day 12, during a 20-day antibiotic treatment regimen), when 76.4% (42 of 55) of patients were positive by ELISA IgG or IgM and 80.0% (44 of 55) were positive by IgG or IgM immunoblot. Since 93% of the seroconversions occurred by V2, it may be of value for physicians to test a patient approximately 2 weeks into treatment, if serology is negative at the patient's first consultation. This may minimize problems later if treatment is unsuccessful or symptoms reoccur by providing serologic confirmation of the earlier diagnosis of Lyme disease.

The length of time antibodies persist will depend on the serological assay used. The ELISA measures antibody concentration, and 1 year after treatment 17.1% (6 of 35) of the IgM ELISA-positive patients and 17.4% (4 of 23) of the IgG ELISA-positive patients remained positive. The immunoblot as routinely used measures only the presence of antibodies, and accordingly more patients remained antibody positive with this assay. One year after therapy, 31.7% (13 of 41) of the IgM immunoblot- and 54.6% (24 of 44) of the IgG immunoblot-positive patients remained positive. Five patients in this study group did not have a satisfactory response to antibiotic therapy, as assessed at V4. Neither the ELISA nor the immunoblot was helpful in identifying these patients. Feder et al. (7) examined the persistence of antibodies in patients with a variety of clinical manifestations (EM, arthritis, and neuropathy) who received appropriate antibiotic therapy. They reported that bands on the IgG immunoblot could be detected as long as 3 years after therapy and that the ELISA and immunoblot were not helpful for identifying patients with persistent or recurrent symptoms.

It has been suggested that early antibiotic therapy may abort the antibody response in patients treated soon after infection (30). Aguero-Rosenfeld et al. (1) reported that 13% (8 of 59) of treated early-disease patients did not seroconvert and two of these patients had culture-positive EM lesions. We found that 20% (11 of 55) of our patients with early Lyme disease did not develop either a positive IgM or a positive IgG immunoblot during the duration of this study. It is possible that the clinical diagnosis of EM for some of these patients was incorrect since culture of the skin was not conducted.

On the basis of our study of 55 clinically and serologically well-defined patients with early Lyme disease, several recommendations for standardization and use of the immunoblot for the serodiagnosis of Lyme disease can be made. (i) MAbs should be available and used for identification of proteins that are of diagnostic importance, since they may vary in molecular weight in different isolates of *B. burgdorferi*. The use of MAbs will allow the comparison of the results of immunoblots from different laboratories. (ii) Since there is variation in the expression of some proteins by *B. burgdorferi* strains, only those that adequately express the proteins of diagnostic importance should be used. (iii) The concentration of acrylamide gel used must be sufficient to allow resolution of proteins of similar molecular masses, such as the 39- and 41-kDa proteins. These two proteins are not well separated on 10% acrylamide gels. (iv) Both IgM and IgG assays should be conducted on sera from patients with early Lyme disease. Most patients with early disease have a good IgG response, and some reinfected patients may have only an IgG response. (v) Patients seronegative at the time treatment is initiated should be retested 2 weeks later if serological confirmation of the clinical diagnosis is desirable.

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